THE NATURE OF THE ACTION OF IONS AS INDUCTORS[1]

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Previous work (Barth and Barth, 1959, 1962, 1963, 1964) demonstrated the action of ions as inductors of various types of nerve, pigment cells and neuroglia. The ions Li^+, Mg^{++} and Ca^{++} were particularly effective and Li^+ was shown to induce a sequence of inductions depending upon its concentration and the duration of exposure. This present work deals with experiments designed to test two of the possible modes of action of Li^+ and other ions. The first test is of a destabilizing action of Li^+ and other ions on proteins and DNA. Von Hippel and Wong (1964) have studied the effects of ions on the melting temperature of ribonuclease and have concluded that the effectiveness of various ions is

$$SCN^- > Ca^{++} > Br^- > Li^+ > Na^+ = K^+.$$

Hamaguchi and Geiduschek (1962) found that the thermal denaturing temperature of DNA is lowered by ions in the order of $SCN^- > I^- > Br^-$, Cl^-. Li^+, Na^+ and K^+ are also effective but with minor differences among the three ions.

The second test was stimulated by the report of Ross and Scruggs (1964) that cations decreased the electrophoretic mobility of DNA in the order $Li^+ > Na^+ > K^+$ and $Mn^{++} \gg Mg^{++} > Ca^{++}$. A comparison of the electrophoretic data with the binding of these ions with long chain polyphosphates "supports the idea that the phosphate groups of DNA are the most likely sites of interaction with the alkali metal ions and the particular divalent ions studied" (Ross and Scruggs, 1964, p. 86).

Since the histone-DNA bond appears to be through phosphate, the thought occurs that ions with affinity for phosphate might possibly release histone from DNA and thus activate DNA. The activation of DNA by release of histone is recorded by Huang and Bonner (1962) and the inhibition of DNA activity by added histone is shown by Huang and Bonner (1962) and Allfrey, Littau and Mirsky (1963). Thus, one possible action of ions as inductors would be to activate DNA by competing with histone for phosphate bonds.

Methods

The methods are the same as described in our previous papers, with four variations. (1) Where relatively high concentrations of an ion (0.1 *M*) were necessary the sodium chloride in our medium was omitted. The sodium chloride in our medium is 0.88 *M*. Therefore, to keep the osmotic pressure at a level consistent with the viability of cells, sodium chloride was omitted in some cases. (2) In order to treat the cells with high concentrations of monovalent cations we used divalent anions such as $SO_4^=$ to keep the osmotic pressure low. (3) In the case of Ca^{++} and

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Mn^{++} we were obliged to omit from our medium the $NaHCO_3$ and the calf serum. Controls treated with our medium lacking $NaHCO_3$ and calf serum differentiated as epithelium. (4) The substitution of lyophilized calf serum (Nutritional Biochemicals Corporation) for globulin from serum (Bios) was made because the latter preparation was no longer available.

A new type of cell is recognized in this study. The cell resembles a unipolar spongioblast and is found in large numbers along with spreading nerve. An entire aggregate may differentiate into these cells. More often there is a mixture of nerve cells and unipolar spongioblasts.

Results

Destabilization of proteins and DNA

Two ions, SCN^- and Br^-, were used to test the possibility that Li^+ was effective as an inductor because of its action as a destabilizer. If so, then SCN^- and Br^- should be more effective than Li^+. LiCl induces a sequence of cell types from nerve to neuroglia at concentrations of from 0.047 *M* to 0.070 *M*. When sodium thiocyanate is added to our medium in concentrations less than 0.05 molar no visible action occurs. If the concentration is increased to 0.074 to 0.084 *M* some spreading nerve is induced but many dead cells result. Thus, possibly the concentration of Na^+ is too high and to test this possibility solutions were prepared with NaCl (0.086 *M*) added. These solutions give the same results as did the addition of 0.084 *M* NaSCN. Therefore the NaCl in the medium was omitted and 0.1 *M* NaSCN added, with the result that the cells survived and differentiated into epithelium and a little nerve. The SCN^- ion is therefore much less effective than Li^+. Similarly NaBr 0.1 *M* if added to our medium produces mostly dead cells, but if substituted for NaCl 0.1 *M* NaBr permits the differentiation of epithelium. In fact the cells do not distinguish between NaCl and NaBr, and cells differentiate normally when cultured in our medium with 0.1 *M* NaBr used in place of 0.088 *M* NaCl. The Br^- ion then is completely inactive at concentrations where Li^+ induces the whole sequence of cell types. The ion series for induction is thus $Li^+ \ll SCN^- > Br^-$, while the series for destabilization is $SCN^- > Br^- > Li^+$.

Comparison of inductive effect and binding of ions to DNA

Ross and Scruggs (1964) studied the effects of ions on the electrophoretic mobility of DNA. For monovalent cations the effectiveness was $Li^+ > Na^+ > K^+$, while for divalent cations it was $Mn^{++} \gg Mg^{++} > Ca^{++}$. At the beginning of the experiments we knew that Li^+ would induce the whole sequence of cellular differentiation from radial nerve to neuroglia. Mg^{++} and Ca^{++} would only induce as far as spreading nerve. Therefore, we tried Mn^{++} to see if it would be more or less effective than Mg^{++} and Ca^{++}.

Table I records the results of experiments in which $MnCl_2 \cdot 4H_2O$ was applied to the presumptive epidermis in varying concentrations and for different intervals of time. Calf serum and $NaHCO_3$ were omitted from the solutions since Mn^{++} precipitates in the presence of these two substances.

In general Mn^{++} has an action similar to that of Li^+ and induces a sequence of cell types depending upon concentration and duration of treatment. In particular

TABLE I

The sequential action of $MnCl_2 \cdot 4H_2O$ on cellular differentiation

In this and succeeding tables the headings are to be interpreted as follows.

Stage no.: Shumway (1940); conc.: concentration of substances in milligrams per milliliter of standard solution; hrs.: time in hours during which aggregates are exposed to the substances indicated; types of cellular differentiation: Barth and Barth 1962, 1963, 1964.

Exp.	Stage No.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11–	0.15	2	40	Epithelium
	11–		3	40	Epithelium
	11–		4	40	Epithelium
	11–		cont.	20	Ciliated masses
2	11	0.5	4	50	Mucous cells
			8	50	Mucous cells, nerve cells
			22	25	Mucous, pigment cells
			30	45	Mucous, pigment cells
3	11–	0.5	2	20	Epithelium, radial nerve
			3.5	20	Epithelium, nerve
			5.5	20	Mucous cells, nerve
4	11	1.0	2	20	Epithelium, radial nerve
			3.5	20	Spreading nerve, epithelium
			5.5	20	Pigment cells, spreading nerve
5	11	1.0	6.0	25	Dead cells, pigment cells
			6.6	25	Dead cells, pigment cells
6	11	1.25	0.1	25	Epithelium, radial nerve, spreading nerve
			1.5	25	Spreading nerve, radial nerve
			2.6	25	Spreading nerve
			3.5	25	Spreading nerve
			4.0	25	Pigment cells
			4.5	25	Pigment cells
7	11–	1.25	1.0	35	Radial nerve, epithelium, spreading nerve
			2.1	35	Spreading nerve, epithelium
			4.3	35	Pigment cells, nerve
8	11+	1.25	2.1	25	Spreading nerve, radial nerve, pigment cells
			2.5	25	Spreading nerve, pigment cells
			3.0	25	Pigment cells, nerve
			3.3	50	Pigment cells, nerve
9	11	1.3	0.0	25	Epithelium
			2.1	25	Epithelium, nerve, pigment cells
			2.6	25	Nerve, pigment cells, mucous cells
			3.4	25	Pigment cells, little nerve
			3.6	25	Pigment cells, mucous cells
			4.0	25	Pigment cells, mucous cells

TABLE I (continued)

Exp.	Stage No.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
10	11	1.3	0.0	25	Epithelium
			2.0	25	Epithelium, mucous cells, nerve
			2.5	25	Pigment cells, nerve, mucous cells
			3.0	25	Pigment cells
			5.2	50	Dead cells, pigment cells
11	11	1.5	0.0	35	Epithelium
			1.0	35	Radial nerve
			2.1	35	Spreading nerve, pigment cells
			3.0	35	Dead cells, pigment cells
			3.6	35	Dead cells
			5.0	35	Dead cells
12	11	2.0	0.0	20	Epithelium
			2.0	20	Pigment cells
			2.8	20	Pigment cells
			6.0	20	Dead cells
			6.6	20	Dead cells

a concentration of 1.25 mg./ml. applied from 0.1 hour to 4.5 hours induces first radial nerve, then spreading nerve and finally pigment cells (Exp. 6, Table I). Lower concentrations induce mucous cells as does Li^{+}. Higher concentrations induce a sequence of cell types but mortality of cells is high. The most effective concentration of $MnCl_2 \cdot 4H_2O$ is about 0.0063 *M*. This compares with a concentration of 0.0016 *M* used by Ross and Scruggs (1964) in their studies on electrophoretic mobility of DNA.

If the phenomena described by Ross and Scruggs (1964) are related to inductive effects, then since Mn^{++} is effective at low concentrations Mg^{++} and Ca^{++} should produce the same effects at higher concentrations if the cells will tolerate them. The addition of $MgSO_2 \cdot 7H_2O$ to our medium in high concentrations (0.1 *M*) resulted mostly in dead cells. However when the NaCl was omitted from the medium the cells would tolerate 0.12 *M* Mg and the sequence of inductions previously obtained by Li^{+} and Mn^{++} was found (Table II, Exp. 5, 6).

The least effective divalent cation in the study by Ross and Scruggs (1964) was Ca^{++}. Although previous investigations (Barth and Barth, 1962) showed that Ca^{++} would induce radial and spreading nerve, no pigment cells were obtained. We, therefore, tested higher concentrations of Ca^{++} and encountered the difficulty that Ca^{++} precipitated in our medium. By omitting calf serum and $NaHCO_3$ we were able to use higher concentrations of Ca^{++} than previously used. In order to keep the osmotic pressure within the limits of viability of cells we also omitted NaCl. The results of the application of various concentrations of $CaCl_2 \cdot 2H_2O$ for varying lengths of time are recorded in Table III. The data show induction of spreading nerve, slate grey epithelium and pigment cells by Ca^{++} at concentrations of 12.0 mg./ml. and 15.0 mg./ml. Thus, Ca^{++} is effective as an inductor at concentrations of from 0.081 to 0.1 *M*. These concentrations are somewhat lower

TABLE II

The sequential action of $MgSO_4 \cdot 7H_2O$

Exp.	Stage No.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11	18.0	1.2	20	Nerve, epithelium
			2.0	20	Spreading nerve
			4.0	20	Dead cells, nerve
2	11	20.0*	2.0	35	Spreading nerve
			3.0	40	Spreading nerve, dead cells
			4.0	35	Dead cells, nerve
3	11	25.0	1.6	35	Spreading nerve, other nerve
			3.0	40	Spreading nerve, UPS
			3.5	35	Spreading nerve, UPS
			4.0	40	Spreading nerve, UPS
4	11	30.0	1.8	25	Spreading nerve
			2.3	25	Spreading nerve
			2.8	25	Spreading nerve, UPS
			3.3	25	Spreading nerve, UPS
			4.0	25	Spreading nerve, UPS
			6.0	25	Spreading nerve, UPS
5	11	30.0	3.1	25	Spreading nerve
			5.0	25	Spreading nerve
			7.7	25	Slate-grey epithelium, nerve, pigment cells
			8.5	35	Slate-grey epithelium, pigment cells, nerve
6	11	30.0	8.3	25	Nerve, slate-grey epithelium
			9.0	50	Spreading nerve, slate-grey epithelium, pigment cells
	11	35.0	3.5	25	Spreading nerve, UPS
			8.3	25	Slate-grey epithelium, nerve
			9.0	25	Slate-grey epithelium, nerve, pigment cells
7	11+	35.0	2.1	25	Spreading nerve, slate-grey epithelium
			5.4	25	Slate-grey epithelium, nerve
			7.8	25	Nerve, pigment cells
			8.0	45	Pigment cells, nerve

* $MgCl_2 \cdot 6H_2O$ used in this experiment.
UPS = unipolar spongioblasts.

than the effective concentrations of Mg^{++} and the series for induction becomes $Mn^{++} \gg Ca^{++} > Mg^{++}$.

In assessing the value of the comparison it must be pointed out that in our studies, Mg^{++} was used in the presence of calf serum and $NaHCO_3$, while Ca^{++} was applied in the absence of these substances. Undoubtedly there was some binding

TABLE III

The sequential action of $CaCl_2 \cdot 2H_2O$

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11−	10.0	2.1	15	Nerve, epithelium
			4.4	15	Nerve
2	11	10.0	1.0	25	Spreading nerve, epithelium
			3.0	25	Nerve, slate-grey epithelium, UPS
			3.8	25	Spreading nerve, slate-grey epithelium, UPS
			8.8	75	Slate-grey epithelium, pigment cells, spreading nerve
3	11	12.0	8.8	40	Nerve, dead cells
			9.8	42	Dead cells
4	11+	12.0	4.8	35	Slate-grey epithelium, UPS, nerve
			7.0	45	Slate-grey epithelium, pigment cells, nerve
5	11	12.0	3.5	35	Spreading nerve, UPS, pigment cells
			6.3	40	Pigment cells, nerve, slate-grey epithelium
			6.6	35	Pigment cells, nerve
			20.0	40	Dead cells
6	11	12.0	5.0	40	Slate-grey epithelium, pigment cells, UPS, nerve
			7.2	42	Pigment cells, slate-grey epithelium, nerve
			7.8	48	Pigment cells, slate-grey epithelium, nerve
			8.4	40	Pigment cells, nerve, dead cells
7	11+	12.0	7.3	25	Spreading nerve, slate-grey epithelium, pigment cells
			22.0	25	Dead cells
8	11	15.0	2.7	25	UPS, spreading nerve
			4.5	25	UPS, spreading nerve
			7.5	25	UPS, spreading nerve
			8.2	75	Spreading nerve, UPS, dead cells
9	11	15.0	3.3	35	UPS, spreading nerve
			4.3	40	UPS, spreading nerve
			4.8	35	UPS, spreading nerve, dead cells
			5.3	40	UPS, spreading nerve, dead celss
10	11	15.0	4.8	35	UPS, spreading nerve
			5.3	40	UPS, spreading nerve
			7.0	35	UPS, spreading nerve
			8.0	40	UPS, spreading nerve

* Not typical.
UPS = unipolar spongioblasts.

TABLE III (continued)

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
11	11+	15.0	7.3	25	Nerve, slate-grey epithelium, pigment cells
			22	25	Dead cells
12	11+	18.0	3.0	40	UPS, spreading nerve
			4.0	42	UPS, spreading nerve
			4.5	40	Spreading nerve, UPS
			5.3	42	Spreading nerve, UPS
			7.4	20	Spreading nerve, UPS, dead cells
13	11	18.0	2.8	25	Radial nerve
			4.2	25	Spreading nerve, Ca^{++}-induced nerve
			5.0	25	*Spreading nerve, UPS
			8.8	25	*Spreading nerve
14	11+	20.0	4.7	40	Spreading nerve, UPS
			7.0	36	Dead cells, spreading nerve
15	11	20.0	2.8	25	Radial nerve
			4.2	25	Spreading nerve, Ca^{++}-induced nerve
			4.0	25	*Spreading nerve, UPS
16	11+	25.0	2.0	25	Radial nerve, spreading nerve
			3.0	25	Spreading nerve, UPS
			3.2	25	Spreading nerve, UPS
			3.5	30	UPS, Spreading nerve
			3.8	30	Dead cells, UPS, nerve
			4.2	40	Dead cells

of Mg^{++} by calf serum at pH 8.0 while there was no such binding possible in the case of Ca^{++} because the calf serum was omitted. This difference in the use of Mg^{++} and Ca^{++} is a fault in the design of the experiments whereby Mg^{++} was used first and when it was found that Ca^{++} would not remain in solution with calf serum and $NaHCO_3$, the latter substances had to be omitted.

With the above comment in mind, there is a close enough resemblance between our studies on induction and those of Ross and Scruggs (1964) to warrant further study. We, therefore, examined the effects of monovalent cations which Ross and Scruggs rate as $Li^+ > Na^+ > K^+$ in relative binding strengths of 1.5 to 1.0 to 0.8. The concentrations used were of the order of 0.02 *M* for Li^+. We find that Li^+ at this concentration induces a sequence of cell types after the cells are exposed for long periods (24–48 hours). Actually it is more expedient to use higher concentrations of Li^+ (0.1 *M*) for shorter periods of time. Therefore, we would need to apply concentrations of from 0.15 *M* to 0.2 *M* Na^+ or K^+ to obtain inductions similar to those of Li^+. Such concentrations attained by either NaCl or KCl are too high for the viability of cells, possibly because of the high osmotic pressure.

TABLE IV

The sequential action of K_2SO_4, Na_2SO_4 and LiCl

Exp. no.	Stage no.	Treatment		No. of aggregates	Types of cellular differentiation
		Conc.	Hrs.		
1	11	8.7K$^+$	4.0	40	Epithelium
			18.0	30	Dead cells, mucous cells
2	11+	8.75K$^+$	4.0	35	Epithelium
			6.0	35	Epithelium, mucous cells
3	11	17.5K$^+$	3.0	25	Radial nerve
			4.0	25	Spreading nerve
			8.0	25	Dead cells, spreading nerve
			8.5	75	Dead cells, rare nerve
4	11+	14.2Na$^+$	4.8	35	Spreading nerve
			5.8	40	Spreading nerve
			6.5	35	Dead cells, spreading nerve
5	11	14.2Na$^+$	4.0	35	Radial nerve, spreading nerve, epithelium
			5.0	40	Spreading nerve, radial nerve
			6.0	35	Spreading nerve
			22.0	35	Dead cells
6	11	20.0Na$^+$	3.0	25	Radial nerve, spreading nerve epithelium
			7.8	25	Dead cells
7	11+	20.0Na$^+$	5.0	25	Nerve, dead cells
			5.7	25	Dead cells, nerve
			6.2	25	Dead cells, nerve
			9.1	25	Dead cells
8	11	4.0Li$^+$	1.3	25	Radial nerve, spreading nerve, epithelium
			2.0	25	Spreading nerve, epithelium, rare pigment cells
9	11	6.0Li$^+$	1.0	25	Spreading nerve, radial nerve, slate-grey epithelium
			2.0	25	Pigment cells, spreading nerve
			4.3	25	Pigment cells
10	11	8.0Li$^+$	1.0	25	Slate-grey epithelium, spreading nerve
			2.0	25	Pigment cells
			4.3	25	Dead cells, pigment cells

In any case we found that the cells would tolerate up to 0.28 *M* Na$^+$ if applied as Na_2SO_4 and up to 0.2 *M* K$^+$ if applied as K_2SO_4.

Table IV compares the effects of K$^+$, Na$^+$ and Li$^+$ added to our medium in which NaCl is omitted. First, it can be seen that K$^+$ and Na$^+$ do not induce the

entire sequence of cell types as does Li^+. K^+ and Na^+ will induce mucous cells, radial nerve and spreading nerve, but not pigment cells. The effectiveness of K^+, Na^+ and Li^+ as regards the induction of spreading nerve is $Li^+ > K^+ = Na^+$. Ross and Scruggs (1964) report Na^+ as slightly more effective than K^+. As regards pigment cell induction, either it is not possible to expose the cells to a concentration of K^+ or Na^+ high enough to induce pigment cells, or the mechanism of induction of pigment cells is not related to the binding power of these ions.

So far as the monovalent cations are concerned, there is evidence of additive effects of any two cations. For example if the complete medium is used containing 0.088 *M* NaCl, then less Li^+ is required for the induction of pigment cells (0.074 *M* as compared with 0.10 *M* when NaCl is absent). Similarly 0.1 *M* K^+ has no effect by itself but if applied in the presence of 0.088 *M* NaCl, induction of radial nerve, spreading nerve and unipolar spongioblasts is obtained.

Discussion

The correlation between the destabilizing effect of various ions on proteins and DNA does not support the hypothesis that ion inductors function by means of a destabilizing effect. However, this finding cannot be construed as evidence against the hypothesis, for there is the question of whether the ions penetrate the cell and reach the DNA. The fact that NaBr will substitute for NaCl in our culture medium may possibly indicate that the Br^- ion does not penetrate.

In addition, the concentrations used for destabilization of DNA were of the order of 1.0–4.0 *M*, whereas the concentrations used to test inductive capacity were of the order of 0.1 to 0.2 molar. We conclude, therefore, that while we have no evidence for the hypothesis we cannot exclude it.

The correlation between the combining power of various ions with DNA and their inductive capacity is a very close one. There is good correspondence of the order of effect of monovalent and divalent cations as regards their inductive effect and their binding with long chain polyphosphates. In addition, the order of magnitude of the concentrations used in both sets of experiments is the same. There still remains the question of the effective intracellular concentration of the ions in the experiments on induction. We do not know the actual concentrations of the ions in the cell nor the distribution of the ions within the cell. Experiments are planned to yield this information.

Granting that the ions penetrate, the combining sites are not known. Long chain polyphosphates other than DNA may combine with the ions. In addition the ions may possibly combine at sites other than the phosphates of DNA and although the amount bound would be much less, nevertheless this type of binding may be the effective one in activating DNA.

The general hypothesis would state that ions act as inductors by competing with histone for phosphate sites on DNA. The resulting dissociation of histone from DNA would progressively activate DNAs.

In evaluating the extent to which the hypothesis applies, we must consider the problem of the action of proteins as inductors. How could they produce an effect by altering phosphate binding? At first sight there seems to be very little in common between the actions of ions and proteins. However, two possible modes of action of the proteins could lead to binding of DNA phosphate. If the proteins

used as inductors penetrate the cell as protein molecules, these would compete with histone for the phosphate sites on DNA or, if acid, proteins would compete with the DNA phosphate for alkaline sites on the histone. In either case dissociation of the DNA-phosphate-histone would be increased.

Vainio, Saxén, Toivonen and Rapola (1962) found that bone marrow proteins penetrated ectoderm cells as tested by their combination with fluorescent antisera. They find no definite concentration of the fluorescent antigen in the nuclei but rather a uniform distribution. Yamada (1962), reporting experiments by Okada and K. Takata, states that fluorescent antisera show an intense staining of the cytoplasm, especially on the surface of yolk granules. Some staining of the nucleus was also observed.

Another possible explanation of the action of proteins as inductors stems from the fact that they are applied to the ectoderm as precipitates. The proteins are soluble in weak salt solutions and would be expected to go into solution. Thus, a saturated solution of protein would be in contact with the cell membranes. It follows that water must be withdrawn from the cells and this would lead to an intracellular concentration of the Na^{+}, K^{+}, Mg^{++} and Ca^{++} ions present. This increase in concentration of ions may possibly be enough to induce nervous tissues by the mechanism of phosphate binding.

In this connection we wish to report some experiments with a 0.17 *M* sucrose solution which induces a sequence of cell types beginning with radial nerve and ending with pigment cells. The effects of 0.17 *M* sucrose are the same as those of Li^{+}, Mn^{++} Mg^{++} and Ca^{++}. A tentative interpretation of the effect of sucrose is offered. Sucrose withdraws water from the cell and as a result the internal concentration of the ions is increased to a point where induction occurs. More work with high molecular weight compounds is necessary to test this idea.

Summary

1. A comparison of the effects of ions as inductors, on the one hand, and on destabilization of proteins and DNA, on the other, shows a lack of correspondence as regards the effectiveness of various ions.

2. A comparison of the effects of various monovalent and divalent cations as inductors with their effects on the electrophoretic mobility of DNA reveals a close correspondence as regards the effectiveness of the ions.

3. An hypothesis of the nature of the effect of cations as inductors is presented. Cations act to combine with the phosphate groups of DNA, thereby removing it from the inhibiting action of histones.

4. A possible interpretation of the action of proteins as inductors by increasing the intracellular concentration of cations is offered.

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